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Gene loops juxtapose promoters and terminators in yeast

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Mechanistic analysis of transcriptional initiation and termination by RNA polymerase II (PolII) indicates that some factors are common to both processes[1,2]. Here we show that two long genes of *Saccharomyces cerevisiae, FMP27* and *SEN1*, exist in a looped conformation, effectively bringing together their promoter and terminator regions. We also show that PolII is located at both ends of *FMP27* when this gene is transcribed from a *GAL1* promoter under induced and noninduced conditions. Under these conditions, the C-terminal domain of the large subunit of PolII is phosphorylated at Ser5. Notably, inactivation of Kin28p causes a loss of both Ser5 phosphorylation and the loop conformation. These data suggest that gene loops are involved in the early stages of transcriptional activation. They also predict a previously unknown structural dimension to gene regulation, in which both ends of the transcription unit are defined before and during the transcription cycle.

The genome of *S. cerevisiae* is highly compact, with 72% comprising open reading frames (ORFs) and an average gene size of 1.4 kb (ref. 3). To distinguish the separate stages of transcription, we investigated two large *S. cerevisiae* genes, *FMP27* and *SEN1*, with ORFs of 7,887 bp and 6,696 bp, respectively. We first determined the transcript profile across *FMP27* by using transcription run-on (TRO) analysis[4] (**Fig. 1**). We observed an accumulation of active polymerase at the 5′ end of the gene, indicating promoter proximal pausing[5], and a second accumulation in the ORF, which finally terminated beyond the poly(A) signal (**Fig. 1b**). We observed a similar TRO profile, though with higher transcription levels, when *FMP27* transcription was driven by the *GAL1* promoter[6] (**Fig. 1c**).

We next carried out chromatin immunoprecipitation (ChIP) analysis on *GAL1::FMP27* with an antibody specific for PolII. The ChIP profile obtained from yeast grown in galactose induction conditions (**Fig. 2a**) showed large amounts of PolII across the gene. Almost no PolII signal was detected under glucose repression conditions, but we obtained an unexpected result with yeast grown in raffinose (non-inducing conditions). In these conditions, the Gal4p transcription factor binds to UAS_G sites in the *GAL1* promoter but is blocked for productive transcription by binding to Gal80p (ref. 7). Despite this block, we observed considerable but asymmetric amounts of PolII

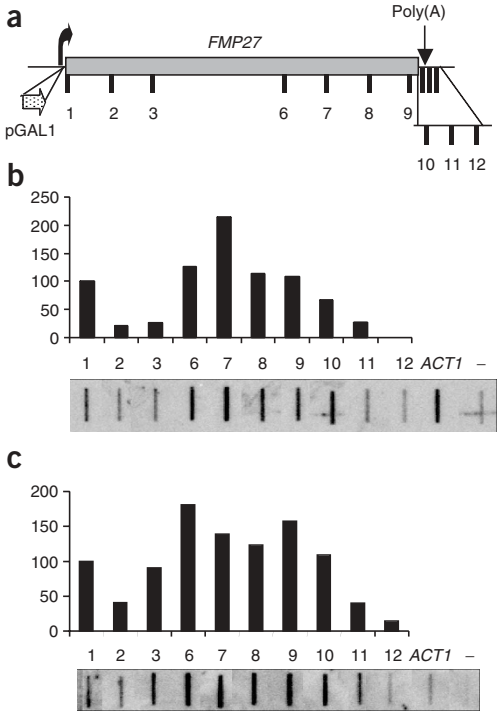

Figure 1 Density of active PolII across the *FMP27* ORF. (**a**) *FMP27* probes (1–12) used in the TRO analysis. Probe positions are listed in **Supplementary Table 1** online. The ORF is depicted by a gray box and the transcriptional start site by a curved arrow. Insertion of the *GAL1* promoter (pGAL1) is also indicated. The poly(A) signal is located in the region between probes 10 and 11 (ref. 10). (**b**,**c**) Density of actively transcribing PolII across the native *FMP27* ORF in wild-type yeast (**b**) or the heterologous *GAL1::FMP27* mutant strain (**c**) measured by TRO analysis. Quantification of TRO signals obtained by phosphorimage analysis is corrected for background (–), uracil content and hybridization efficiency. The relative transcription of endogenous actin detected by the *ACT1* probe indicates that *GAL1::FMP27* is transcribed at higher levels than endogenous *FMP27*.

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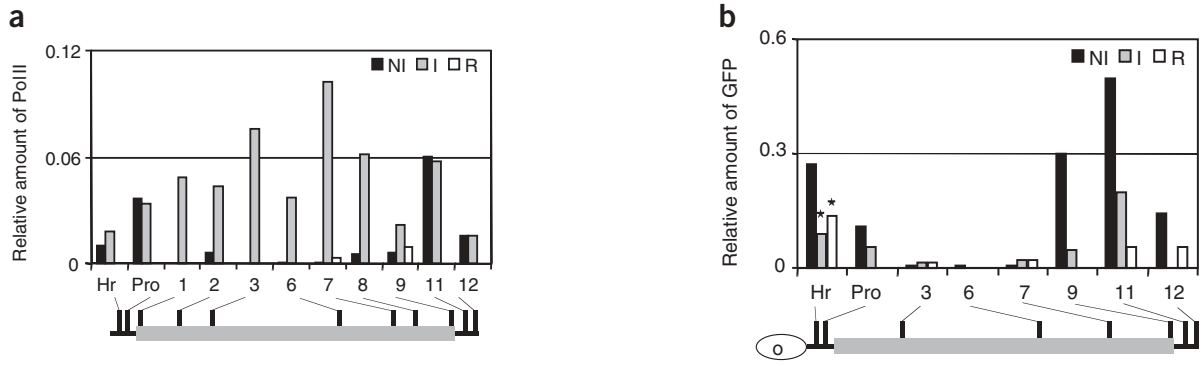

Figure 2 PolII localizes in the promoter and terminator regions of *GAL1::FMP27*, causing looping of the intervening ORF. (**a**) *GAL1::FMP27* cells were grown in raffinose, crosslinked with formaldehyde and then treated for 10 min with raffinose (noninduced, NI), galactose (induced, I) or glucose (repressed, R). Chromatin was immunoprecipitated with antibody to PolII and PCR was done with the indicated primers (see **Supplementary Table 2** online). Immunoprecipitations were quantified and are expressed relative to the input. (**b**) *lac*O-*GAL1::FMP27* cells expressing *HIS3:lacI-GFP* were induced with 3-aminotriazole and then subjected to *GAL1::FMP27* induction and ChIP analysis as in **a**, except that antibody to GFP was used. The position of *lac*O upstream of the Hr region is indicated. The asterisk indicates that reduced (75% of normal) sonication conditions were used.

over the promoter and terminator regions, though not in the ORF. The amount of PolII also dropped upstream of the promoter in region Hr and downstream of the terminator in region 12 (**Fig. 2a**).

These results suggest that the promoter and terminator regions exist in close spatial proximity under noninducing conditions. To test this hypothesis, we tagged *GAL1::FMP27* with an upstream *Escherichia coli lac* operator in a strain coexpressing a fusion protein of the Lac repressor and green fluorescent protein (GFP; **Fig. 2b**). Under noninducing conditions, ChIP analysis using antibodies to GFP detected the promoter region and, notably, the terminator region, but not the ORF (**Fig. 2b**). The signal from the terminator region could result only from crosslinking between the promoter and terminator regions, which therefore must be in close spatial proximity.

We also detected substantial signals at both ends of the gene under inducing conditions, indicating a looped gene structure. As expected, we detected signals over the Hr region adjacent to *lac*O under all three growth conditions. For the repressing and inducing conditions, however, less-stringent sonication conditions were necessary to detect signals. The Hr region may be sensitive to sonication, but under noninducing conditions the loop complex may extend towards the *lac*O region, protecting the linking DNA from fragmentation.

We obtained independent data for the existence of a gene loop structure using the chromosome conformation capture (3C) technique[8]. In this analysis, chromatin is crosslinked by formaldehyde; fragmented by restriction enzymes; diluted to limit random, intermolecular interactions; and then ligated to covalently join DNA fragments that are crosslinked to the same complex (intramolecular ligation). These ligation products reflect crosslinking between otherwise separate restriction fragments and, with the appropriate primers, are detected by PCR. DNA sequence analysis then confirms that the PCR products derive from intramolecular ligation between fragments with compatible restriction ends.

We carried out 3C analysis of *GAL1::FMP27* under noninducing, inducing and repressing growth conditions (**Fig. 3**), using *Eco*R1 to digest the chromatin preparation. Positive control lanes showed the PCR products expected from the intermolecular ligation of

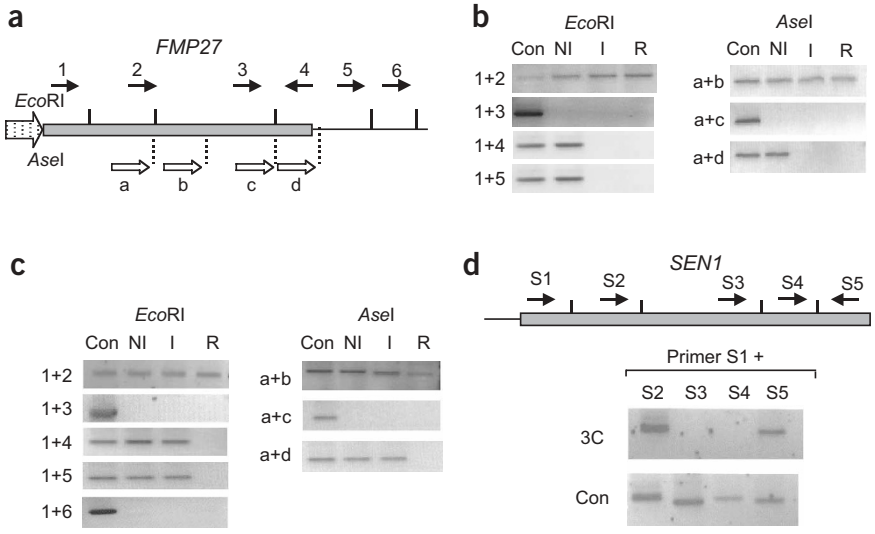

Figure 3 *GAL1::FMP27* and *SEN1* exist in a loop conformation dependent on the expression state. (**a**) *GAL1::FMP27*, illustrating the *Eco*RI and *Ase*I cleavage sites, the primers used in 3C analysis and the *GAL1* promoter (dotted arrow). (**b**) 3C analysis of *GAL1::FMP27*. *GAL1::FMP27* cells were treated with raffinose (noninduced, NI), galactose (induced, I) or glucose (repressed, R). *Eco*RI or *Ase*I and the primer pairs indicated were used for 3C analysis[8]; Con denotes the genomic DNA intermolecular ligation control. PCR products associated with intramolecular ligation were sequenced, and the size and sequence of the PCR products obtained with primers of opposite polarity were consistent with intramolecular ligation between the restriction fragments. (**c**) 3C analysis as in **b**, except that chromatin was crosslinked in whole cells before the nuclei were extracted. (**d**) 3C analysis as in **b**, except that the target gene was *SEN1* and the expression conditions were limited to growth in raffinose. 3C denotes intramolecular ligation PCR products.

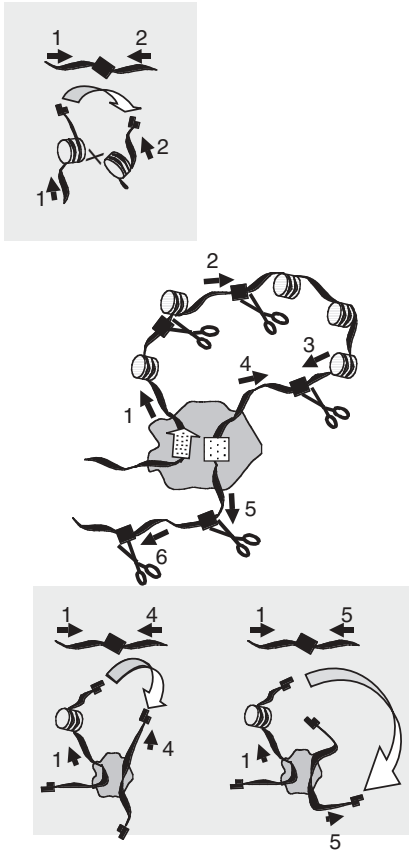

Figure 4 Model of the proposed *GAL1::FMP27* gene loop. The DNA sequence is drawn as a ribbon. The promoter (spotted arrow) and terminator (spotted box) are contained in a diffuse structure, with the ORF of the gene 'looped out'. The positions of nucleosomes are illustrative and not drawn to scale. The *Eco*RI sites (black box with scissors) and adjacent primers are also shown. Insets above and below the gene loop model show how intramolecular ligation can generate the PCR products detected with primers 1 and 2, primers 1 and 4 and primers 1 and 5. X denotes a crosslink between adjacent nucleosomes. Curved arrows indicate interaction and subsequent intramolecular ligation between the *Eco*RI ends of DNA fragments.

*Eco*RI-digested genomic DNA. Primers 1 and 2 from adjacent restriction fragments generated a PCR product indicative of intramolecular ligation under all growth conditions (**Fig. 3b**). Crosslinking between adjacent nucleosomes present on actively transcribed *FMP27* (refs. 9,10) allowed the formation of this intramolecular ligation product (**Fig. 4**).

The absence of PCR product with primers 1 and 3 under all conditions indicated that these nonadjacent *Eco*RI fragments did not ligate and consequently were not in close spatial proximity. By contrast, primers 1 and 4 and primers 1 and 5 both produced products indicative of intramolecular ligation only under noninducing conditions. Presumably, these DNA fragments are held in close proximity through a complex at the base of the proposed gene loop (**Fig. 4**). The product from the oppositely orientated primers 1 and 4 lacked two internal *Eco*RI fragments, again indicating that intramolecular ligation had occurred.

Analysis with a second restriction enzyme, *Ase*I (**Fig. 3**), confirmed that adjacent *Ase*I fragments underwent intramolecular ligation (primers a and b), whereas nonadjacent fragments did not ligate (primers a and c). The more distant fragments generated intramolecular ligation products detected by primers a and d. These data show that *GAL1:FMP27* exists in a loop conformation under noninducing conditions (**Fig. 4**), as predicted from the GFP-targeted ChIP experiment (**Fig. 2b**). In particular, the intramolecular ligation products detected by primers 1 and 4, primers 1 and 5 and primers a and d are all compatible with a complex forming between the promoter and terminator regions (**Fig. 4**). Notably, when the 3C technique was altered so that whole cells were treated with formaldehyde before chromatin extraction, intramolecular ligation products were detected with primers 1 and 4, primers 1 and 5 and primers a and d under both noninducing and inducing conditions (**Fig. 3c**).

Because the ligation products were undetected in repressing conditions, the gene loop conformation is not a consequence of DNA curvature. Instead, we predict that gene loop formation is connected to the transcriptional status of the gene (**Fig. 5**). These data agree with those from the *lac*O ChIP analysis (**Fig. 2b**), which imply that *GAL1::FMP27* is in a looped conformation under inducing conditions. To define this loop structure further, we used an additional primer pair (primers 1 and 6), but this pair gave no ligation product, indicating that the loop structure does not extend beyond the terminator region. These data are consistent with those from the *lacO* ChIP analysis (**Fig. 2b**), which also indicated that the loop structure was restricted to the end of *FMP27*.

Similarly, 3C analysis on *SEN1* (**Fig. 3d**) showed that adjacent *Eco*RI sites resulted in intramolecular ligation products (detected by primers S1 and S2), whereas the nonadjacent *Eco*RI fragments (primers S1 and S3 and primers S1 and S4) did not. Notably, we detected a ligation product with primers S1 and S5 that lacked the three internal *Eco*RI fragments, indicating that these distant *Eco*RI fragments underwent intramolecular ligation. This finding provides evidence for a loop structure between the promoter and terminator regions of *SEN1*. 3C analysis on the endogenous *FMP27* gene driven by its natural promoter showed intramolecular ligation between terminal fragments, indicating that wild-type *FMP27* also exists in a looped conformation (data not shown).

Transcription elongation is coordinated by differential phosphorylation of the PolII C-terminal domain (CTD) heptad repeat at Ser5 and Ser2 (by the Kin28p and Ctdk1 kinases, respectively) with phosphorylation at Ser5 present early and at Ser2 present later in elongation[11]. We did ChIP analyses on *GAL1::FMP27* with antibodies specific for phosphorylated Ser5 and phosphorylated Ser2 (**Fig. 5a**,**b**). As expected, the Ser5 phosphorylation signal was high over the promoter and dropped to lower levels in the ORF, whereas the Ser2 phosphorylation signal was detectable only over the termination region under inducing conditions. Notably, the promoter- and terminator-specific PolII signals (**Fig. 2a**) obtained under noninducing conditions were derived from PolII phosphorylated at Ser5. Thus, PolII is located in an elongation-competent mode at both ends of the gene (**Fig. 5a**). We also observed some PolII phosphorylated at Ser5 in the termination region of *GAL1::FMP27* under inducing conditions.

We finally investigated whether the gene loop is dependent on CTD phosphorylation by using Ser5 kinase (Kin28p) temperature-sensitive mutants[12]. As expected, ChIP analyses detected very little PolII phosphorylated at Ser5 on *GAL1::FMP27* in the *kin28*$^{ts\text{-}4}$ strain (NKY 4044) grown under restrictive conditions (**Fig. 5c**). In addition, Kin28p was localized to both ends of the gene under noninducing conditions (**Fig. 5d**). When we re-examined the PolII profile over *GAL1::FMP27* in the *kin28*$^{ts\text{-}4}$ as compared to wild-type strains grown under restrictive conditions, we obtained a marked, deregulated PolII profile under both noninducing and inducing

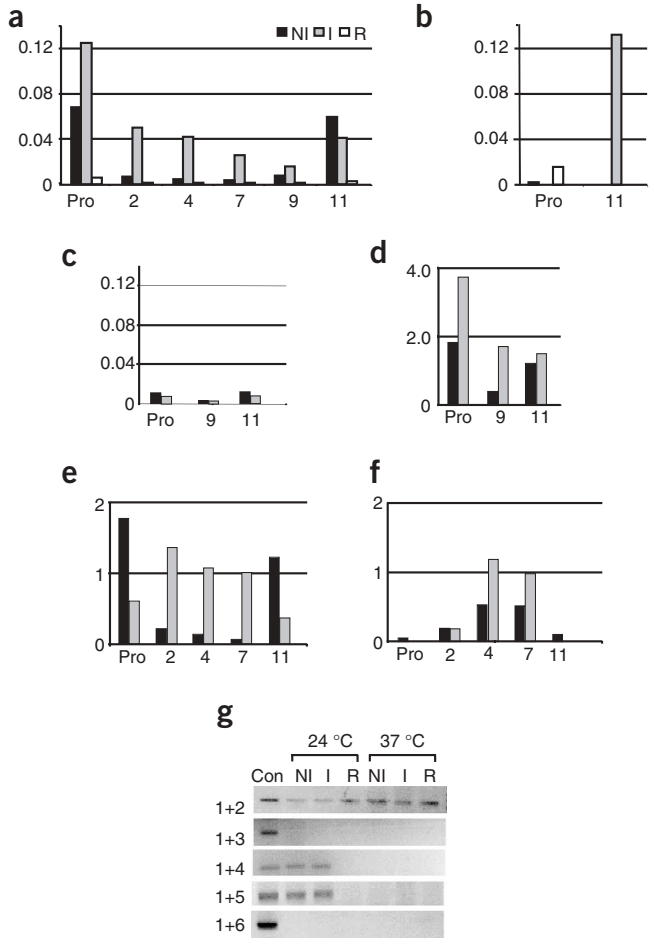

Figure 5 Phosphorylation at Ser5 of PolII CTD is required for gene loop conformation. (**a**–**f**) ChIP analysis of *GAL1:FMP27* as in **Figure 2a**, but using the following antibodies, strains and growth temperatures. (**a**) Antibody to CTD phosphorylated at Ser5 in wild-type strain at 30 °C. (**b**) Antibody to CTD phosphorylated at Ser2 in wild-type strain at 30 °C. (**c**) Antibody to CTD phosphorylated at Ser5 in *kin28*$^{ts\text{-}4}$ strain at restrictive temperature (37 °C). (**d**) Antibody to Kin28p in wild-type strain at 37 °C. (**e**) Antibody to PolII in wild-type strain at 37 °C. (**f**) Antibody to PolII in *kin28*$^{ts\text{-}4}$ strain at restrictive temperature (37 °C). ChIP signal levels are not comparable between antibodies. Positions of PCR products are shown in **Figure 2a**. (**g**) 3C analysis of *GAL1:FMP27* as in **Figure 3c** using *kin28*$^{ts\text{-}4}$ strain grown under permissive (24 °C) and restrictive (37 °C) temperatures.

growth conditions (**Fig. 5e**,**f**). PolII accumulated in the ORF but not in the promoter or terminator regions.

These data suggest that Kin28p kinase activity, which phosphorylates components of the PolII transcription complex including Ser5 in the CTD, is required for gene loop formation. We therefore carried out 3C analysis of *GAL1::FMP27* using the *kin28*$^{ts\text{-}4}$ mutant strain under both permissive (24 °C) and restrictive (37 °C) temperature conditions (**Fig. 5g**). Because all previously defined intramolecular ligation products (obtained with primers 1 and 4 and primers 1 and 5) were absent at 37 °C, we suggest that this gene loop structure is dependent on the initial transcriptional activation of PolII by Kin28p (ref. 12).

Our results provide an explanation for some apparently contradictory observations. For example, the transcription factor TFIIB is associated with Ssu72p (refs. 13,14) and Sub1p (ref. 15), both of which are also involved in mRNA 3′ formation. Similarly, the cleavage polyadenylation specificity factor is a component of TFIID[16]. These observations led to the proposal that some factors that are required for mRNA 3′-end formation load onto the gene at the promoter and then 'piggy-back' on the PolII elongation complex across the gene to the terminator region. By contrast, ChIP analysis on several *S. cerevisiae* genes showed that cleavage or polyadenylation factors are associated only with the terminator regions[17].

We suggest that the apparent duality of some factors in mRNA 3′-end processing and transcription may be explicable by gene looping (**Fig. 4**). The observations that Kin28p is physically present in the loop complex and that its activity is required for loop formation suggest that gene loops are associated with early transcriptional activation. Gene loops may function to proofread control sequences before efficient elongation, or they may be precursors to a reinitiation scaffold[18]. Notably, the alterations in the loop structure that were observed when *GAL1::FMP27* gene was induced could reflect modifications to this scaffold after the first round of transcription.

Genome-wide analysis of gene expression in *S. cerevisiae* shows that adjacent genes often share regulatory sequences, indicating the presence of chromosomal expression domains[19,20]. We predict that, whereas single-gene loops may exist for larger genes such as *FMP27* and *SEN1*, smaller genes may form multigene loops encompassing adjacent genes. Similarly, the periodic organization of the yeast genome is consistent with loop structures that facilitate the dynamic association of coregulated genes in three dimensions[21]. In mammals, much larger genes may possess more complex loop structures. The locus control region of the β-globin gene cluster forms a loop structure with downstream globin gene promoters[22,23]. This locus control region mediates transcriptional activation by promoting phosphorylation of PolII at Ser5 in the CTD at the β-globin gene promoter, rather than by setting up the preinitiation complex[24]. These studies resonate with the *GAL1::FMP27* gene loop structure, which is associated with phosphorylation of PolII at Ser5 in the CTD and dependent on Kin28p activity. We suggest that gene loops may be a common feature of gene activation by promoting efficient transcriptional elongation.

METHODS

Strains and plasmids. *S. cerevisiae* W303-1a *GAL1:FMP27* (originally called *YLR454w*) and YIpplac204 *GAL1:FMP27* (ref. 6) were gifts from K. Struhl (Harvard Medical School). YIpplac204 *lac*O-*GAL1::FMP27* was constructed by cloning an *Eco*RI fragment from pAT12 (ref. 25) into YIpplac204 *GAL1::FMP27*. We constructed *S. cerevisiae* W303-1a *lac*O-*GAL1::FMP27* by transforming the *Kpn*1-linearized YIpplac204 *lac*O-*GAL1::FMP27* into *S. cerevisiae* W303-1a and selecting transformants for tryptophan prototrophy. *S. cerevisiae* W303-1a *lac*O-*GAL1::FMP27* was then transformed with *Nhe*1-linearized pAFS78, and histidine prototrophs were selected to yield *S. cerevisiae* W303-1a *lac*O-*GAL1::FMP27 HIS3*:*lac*I-*GFP*. In NKY 4044 (*Mat a, ura3, leu2, lys2, kin28*$^{ts\text{-}4}$ *TRP::pGAL1:FMP27*), the Kin28p C-terminal mutations are D265N and M270I.

Growth conditions. We maintained strains on synthetic complete (SC) medium containing the appropriate amino acids supplements and glucose (2 % w/v). For ChIP and 3C analysis, we cultured strains to 1.32×10^7 cells per ml in SC raffinose (2% w/v); induced them by dilution with an equal volume of prewarmed (30 °C) SC containing 4% (w/v) glucose (repressed), raffinose (noninduced) or galactose (induced); and incubated them for 10 min (30 °C, 170 rpm) before crosslinking. For targeted ChIP, we grew *S. cerevisiae* W303-1a *HIS3*:*lac*I-*GFP lac*O-*GAL1::FMP27* overnight in SC minus histidine. Before selective induction by carbon source, LacI-GFP was induced by the addition of 10 mM 3-aminotriazole (30 min). The NKY 4044 strain was grown initially for 30 min in raffinose and then for 12 min in galactose at 24 °C for permissive expression and at 37 °C for restrictive expression of Kin28p.

TRO analysis. We labeled and detected nascent transcripts by TRO essentially as described[4]. We designed *in vitro*–transcribed antisense RNA probes (160 ± 10 nucleotides) at intervals across the *GAL1::FMP27* ORF (**Supplementary Table 1** online). We quantified signals for each probe using a phosphorimager and normalized them for uracil content and hybridization efficiency, which were determined by hybridization to synthetic *in vitro*–transcribed RNAs. The background probe was *in vitro*–transcribed pGEM polylinker RNA. Signals above background are shown relative to a 100% signal for probe 1.

ChIP. We did ChIP as described[26] with the following modifications. Crosslinking times were reduced to 6 min for all assays. We used antibodies specific for PolII (H224; Santa Cruz Biotechnology), GFP (G6539; Sigma), PolII phosphorylated at Ser5 in the CTD (H14; Covance), PolII phosphorylated at Ser2 in the CTD (H5; Covance) and Kin28p (Babco). After eluting them from sepharose beads, we treated samples with proteinase K and reversed the crosslinks[27]. We purified templates for real-time PCR by a QIAquick PCR purification kit (Qiagen) and eluted them with water (200 µl).

Real-time PCR. We carried out real-time PCR on a Corbett Rotor-gene 3000 with a QuantiTect SYBR Green kit (Qiagen) and primers (**Supplementary Table 2** online) at concentrations of 0.5 µM. We diluted input DNA between 1:5 and 1:10 and no antibody or and ChIP samples to 1:2 and 1:3, respectively, to ensure the results were linear. PCR conditions were standardized to 45 cycles of 94 °C for 30 s, 57.5 °C for 30 s and 72 °C for 30 s. Results were analyzed as described[26].

3C analysis. We did the 3C analysis as described[8]. Chromatin crosslinking in whole cells was achieved by treating the cells with formaldehyde (1% v/v) for 2 min before extracting the intact nuclei. PCR conditions were standardized to 35 cycles of 94 °C for 1 min, 49 °C for 1 min and 72 °C for 1 min, followed by 72 °C for 7 min. For *SEN1* PCR, the annealing temperature was altered to 54 °C. Details of the PCR primers used for the 3C analysis of *FMP27* and *SEN1* are available on request.

Note: Supplementary information is available on the Nature Genetics website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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